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Use of the Mixed Flask Culture (MFC) Microcosm Protocol to Estimate the Survival and Effects of Microorganisms Added to Freshwater Ecosystems

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16. ABSTRACT

The ability to manipulate an organism's genetic substance offers benefits to many aspects of human health and well-being. Coupled with this positive aspect of genetic engineering, however, is a concern about potential adverse effects on human welfare and environmental quality. Live microorganisms present a special problem when assessing their effects in natural systems. Some determination of the probability of the test organisms' survival in the wild must be made and laboratory microcosms offer an appropriate solution.

This report describes a microcosm test procedure based on the concept that many ecosystem processes are independent of species composition. As long as suitable groups are represented in the microcosms, these processes will occur and a viable, self-sustaining community will develop. Using the procedures outlined in this protocol we have completed 3 tests of chemicals and 4 tests with microorganisms. The report presents the procedures and provides examples of data from our experiments. Complete protocol and data are available from the senior author.

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USE OF THE MIXED FLASK CULTURE (MFC) MICROCOSM PROTOCOL TO ESTIMATE THE SURVIVAL AND EFFECTS OF MICROORGANISMS ADDED TO FRESHWATER ECOSYSTEMS

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I. INTRODUCTION

The ability to manipulate an organism's genetic substance offers opportunities to benefit many aspects of human health and well-being. Coupled with this positive aspect of genetic engineering, however, is a concern about potential adverse effects on human welfare and environmental quality. Some opponents of genetic engineering have been quick to paint images of new organisms running amok and taking over the planet. While this is unlikely, there is legitimate cause for concern over the effects of releasing novel organisms into natural systems. Experience with exotic species which have successfully exploited new niches underscores a need for caution.

New microorganisms are being developed for many purposes. The industrial applications for novel organisms appear boundless; ranging from manufacturing, to pharmaceutical, to agricultural, to mining, to bioremediation. While many of these organisms are designed for use in processes where they can be controlled, others are targeted specifically for release into the environment. One such group, microbial pest control agents (MPCAs), have become the subject of intense development and serve as an example of the test requirements needed for the regulatory process.

Insect pest control has moved from the exclusive use of synthetic organic chemicals to an integrated program of chemicals and pathogenic microorganisms. Originally, naturally occurring pathogens such as Bacillus thuringiensis, Bacillus popilliae, and certain baculoviruses

(4) were used. Low survival and virulence often offset the advantage of host specificity for natural MPCAs. Engineering techniques are being applied to a variety of entomopathogenic bacteria, fungi and viruses in an effort to produce "new organisms" with enhanced pest control attributes missing from the natural organism.

The Environmental Protection Agency (EPA) has responsibility for regulating distinct aspects of the production and use of these genetically engineered microorganisms (GEMs) under two legislative Acts. The Federal Insecticide, Rodenticide and Fungicide Act (FIFRA) directs the EPA to register microbial pest control agents (MPCAs) before they can be used in this country. The Toxic Substances Control Act (TSCA) provides EPA with jurisdictions over all chemicals already in commerce or new chemicals intended for commercial use that are not specifically covered by other regulatory authorities (17). Both Acts give EPA the power to prescribe tests and data-reporting requirements for GEMs or MPCAs. Most tests currently available were developed to provide information for chemicals, however, and may not be directly applicable to predicting impacts of GEMs.

Both FIFRA and TSCA regulate through several procedures including an ecological assessment of the risk associated with using a material. For example, risk assessment for a new chemical pesticide would include some measure of its toxicological hazard determined through a tier testing process and an estimation of the potential environmental exposure of non-target populations (18). Toxicological hazard data, for chemicals in freshwater, would include a suite of tests that produces acute, 2 or 4 day exposure laboratory data usually

in the form of LC50 values. These tests might be followed with more complex life-cycle invertebrate and fish exposures to measure survival and reproduction. Such traditional LC 50 values and reproductive studies may have less value in GEM or MPCA risk assessments because direct lethality and reproduction effects may not be the major environmental impacts. Environmental exposure is determined using a combination of the estimated amount of chemical in and available to organisms and the numbers, types, distribution, abundance, dynamics and natural history of organisms that will be exposed to a chemical. Estimating environmental concentrations and predicting the effects of microorganisms on the structure and function of an ecosystem may be more difficult due to our limited knowledge of the fate and effects of microorganisms introduced into freshwater ecosystems.

Live microorganisms present a special problem when assessing their effects in natural systems. In any controlled testing paradigm the question of containment is critical. Since field tests will result in the release of the test organism they should be used only as the final stage in hazard assessment. Some determination of the probability of the test organisms' survival in the wild must be made before field studies can be used. These considerations point to the need for an intermediate step, a test protocol that is more ecologically realistic than an acute or chronic single species test but more contained than an outdoor experiment. Laboratory microcosms offer an appropriate solution.

Although many types of laboratory microcosms have been used for chemical testing (3; 5; 6; 7; 10; F.B. Taub and P. Read, Standardized

Aquatic Microcosm Protocol. Final Report, Vol. II. FDA Contract 223-80-2352, 1982; P.M. Van Voris, P., M. F. Arthur, and D.A. Tolle, Terrestrial soil core microcosm test protocol and technical support document. Draft Document submitted to the Office of Toxic Substances, U.S. Environmental Protection Agency, Washington, D.C., 1984.) their suitability for evaluating microorganisms effects has yet to be determined. For routine testing, a microcosm should contain a community capable of performing the important trophic interactions seen in the field. The system should be complex enough to allow for decomposition, nutrient cycling, primary production, and contain at least one level of consumers. Yet the system must not be so complex as to be impossible to reproduce. While it is important to measure effects on both ecosystem function and structure, a balance must be struck between the information provided and the cost. Additional considerations include the suitability of the system for containment and decontamination. In general, small, static systems appear to be more easily contained and generate less waste than large or flow-through systems. Small systems also have the advantage of permitting a larger number of replicates, thereby increasing statistical power.

The size of the test system determines, in part, which species can be included. Most microcosms, even large ones, do not support a top predator (e.g. a fish). As the size of the system decreases, the size and numbers of organisms it can support also decreases. Systems containing only a few liters may be unable to support populations of large insect predators. Aquatic systems of less than a liter may be unable to sustain zooplankton populations. Test tube based systems might only support

a microbial community. Each microcosm has advantages and disadvantages. This chapter describes a freshwater microcosm that we believe offers a good compromise on size and ecological complexity.

II. THE TEST SYSTEM

The microcosm test procedures we have developed (L.J. Shannon, T.E. Flum and J.D. Yount.1987. Draft Protocol for a Mixed Flask Culture Aquatic Microcosm Toxicity Test. 34p., 1987) are based on a protocol originally developed by Leffler (7). Although we have extensively modified a number of procedures and added many new ones, the philosophy behind this protocol and the basic approach are still those of Leffler. The test system is a generic one, predicated on the idea that many ecosystem processes (e.g. decomposition, nutrient cycling, primary production, respiration) are independent of species composition. As long as suitable groups are represented in the microcosms, these processes will occur and a viable, self-sustaining community will develop.

We began work with this protocol in 1984 and conducted a series of tests designed to compare the hazard rank of chemicals generated by community and ecosystem level tests with those generated by single species tests (3,20). This work included 16 experiments with the Leffler protocol. During our work, other investigators completed 8 experiments with this method and evaluated the developmental assumptions of the system (16; F.S. Stay, A. Katko, C.M. Rohm, M.A. Fix, and D.P. Larson, Arch.

Environ. Contam. Toxicol., in press) (Table 1).

These experiments showed that Leffler's protocol produced viable, functioning aquatic communities. Modifications were necessary, however, to improve the replicability and repeatability of the test and enhance the procedures used for monitoring, data handling and data analysis. In developing appropriate modifications, we completed 4 additional tests, which resulted in a new draft protocol for the mixed flask culture (MFC) microcosm test (Shannon et al., 1987; USEPA project report. Environmental Research Laboratory-Duluth No. 7667A). Using the procedures outlined in this new protocol we have completed 3 chemical tests and 4 tests with microorganisms (Table 1). The following sections briefly describe the procedures and provide examples of applications where the method has been used. The complete protocol is available from the senior author.

Design Features. This protocol can provide data on the fate and effects of chemicals or survival and effects of microorganisms introduced into a freshwater environment. The system is designed as a generic model of small eutrophic ponds. It contains stable communities of primary producers, consumers and decomposers and changes are monitored at both the population and ecosystem levels. Measurements of pH and dissolved oxygen are used to estimate primary production and respiration. These functions are influenced by factors such as microbial decomposition activities, nutrient cycling, and grazing of protozoans, zooplankton, other crustaceans and insects. As such they provide an integrated measure of effects. Although these

functional measurements are sensitive indicators of perturbation, structural measurements (i.e. population densities) are also necessary to explain the cause of changes observed at the ecosystem level (16).

Microcosms are constructed in one liter pyrex beakers and are covered with a 150 mm x 15 mm plastic or glass petri dish cover. Because of the beaker lip, the system is not completely sealed and gas exchange with the atmosphere can occur. The cover reduces the probability of outside contamination and loss of the introduced agent to the outside. These microcosms contain a defined nutrient medium, a sand sediment and a diverse community of bacteria, fungi, protozoa, algae, rotifers, planktonic and benthic grazers. The starting community "seed" is derived from a variety of natural sources that are allowed to "co-adapt" in the laboratory. The microcosms' small size permits a large number of them to be held in a small space. We have used as many as 5 replicates in each of 6 different treatments or 30 microcosms in a test.

Test Protocol

Development of a stock community. The stock culture is developed in a 40 L aquarium held in an incubator or constant temperature room at 20^{o}C and a 12 hr. light/dark cycle with enough light to support algal growth . The aquarium is first acid washed and carefully rinsed. Acid washed white quartz sand is added to a depth of 2-3 cm and the aquarium is filled with approximately 36L of T82 nutrient medium (Taub and Read, Standardized Aquatic Microcosm Protocol. Final Report, Vol. II.

FDA Contract 223-80-2352, 1982). Samples taken from natural ecosystems are collected and added to the aquarium to start a stock culture. A variety of sites should be sampled in order to ensure a diversity of species in the stock cultures. Suitable sources include small ponds, lakes, marshes, vernal pools or other standing water sources which have no known exposure to toxicants. Approximately 2L of material collected from natural ecosystems should be used to inoculate the stock aquarium.

The stock community is allowed to mature for at least three months prior to use as an inoculum. This maturation period provides a relatively stable species assemblage since those species which are unable to coexist with other members of the community are eliminated, leaving a the "co-adapted" group of species. At least one new stock culture should be prepared every six months to ensure a healthy, growing inoculum. Inoculum from older stock cultures in addition to new "wild" material should be used to start a new culture. Stock cultures may be used for several years unless obvious changes suddenly occur in the system.

Before use, the stock culture should be microscopically examined to determine its species composition. The following minimum criteria must be met:

(a) two species of single-celled green algae or diatoms.
(b) one species of filamentous green alga
(c) one species of nitrogen-fixing blue-green alga.
(d) one grazing macroinvertebrate.
(e) one benthic, detritus-feeding macroinvertebrate.
(f) bacteria and protozoa species.

In our experience, species diversity is typically far greater

than this minimum.

Microcosm construction. Microcosm vessels must be scrupulously clean. We recommend that they be washed five times with tap water, five times with a solution containing 200 mg/L hypochlorite, five times with 10 percent HCl, and five times with distilled water. Microcosms are constructed by adding 50 mL of acid washed quartz sand sediment and 900 mL of Taub #82 medium (supplemented with 15 ug $NaHCO_3$/L as an additional carbon source) to the microcosm vessel. These microcosms are then inoculated with 50 mL of the stock community.

Our tests typically have four treatment groups (a control and three test groups) each containing five replicate microcosms for a total of 20 beakers. Microcosm vessels are incubated at 20°C ± 1°C in a suitably-sized growth chamber on a 12 hour light-dark cycle. Each microcosm is randomly assigned a number and letter which is marked on a piece of tape positioned at the 1-L level on the outer surface of the beaker. After set-up and inoculation, all are placed in the incubator in a randomized block design. The available shelf space is divided into a number of blocks equal to the number of replicate groups. All the "A" replicate are then arranged in one block, the "B" replicates in another. Each block is moved to a new position twice a week. This procedure is necessary to distribute any effects of shelf position. To simulate naturally occurring immigration and permit the re-introduction of extirpated species, all microcosms are reinoculated each week with 10 mL. of the stock community.

Microcosms are allowed to mature for six weeks prior to treatment and are monitored through a six week exposure period. During the first six weeks, consistency among replicates is improved with a weekly cross-inoculation. This is accomplished by thoroughly mixing all microcosms and removing 100 mL from each. These 100 mL aliquots are combined in a 4-L beaker, thoroughly mixed and redistributed among the vessels.

It is prudent to start more systems than needed for the experiment. The extra systems provide replacements for vessels which are accidentally broken or simply fail to develop. Just prior to treatment, beginning measurements are taken and each microcosm evaluated. To be acceptable for use in a test, a microcosm must (1) contain all the required functional groups, and (2) meet minimum criteria of 4 mg/L pre-dawn dissolved oxygen and 11 mg/L late afternoon dissolved oxygen. Any not meeting these criteria are removed. If the remaining group of microcosms is larger than the number needed for a test, those microcosms showing the greatest deviation from the group means for oxygen and pH measurements are successively removed until an appropriate number remain. This process serves to reduce variability among the group of test microcosms.

Variables Measured. During the exposure, monitoring is done on a schedule of decreasing frequency. Measurements are taken twice weekly for the first 2 weeks, and then weekly. Normally the exposure period lasts for six weeks, although longer exposure times can be used.

Functional Variables. Dissolved oxygen (DO) and pH are used as indicators of effect. They are both easily measured and they reflect trophic interactions within the bio-geochemical matrix of the ecosystem (13). By measuring oxygen content three times, just prior to lights, just prior to lights out and just prior to lights on the next morning, diel oxygen gain and loss can be calculated (9). Oxygen gain is a measure of net primary production, while oxygen loss is a measure of community respiration. From estimates of production (P) and respiration (R), a P/R ratio can be computed as a further indicator of the status of the system.

Dissolved oxygen concentrations are measured with a meter and polarographic oxygen electrodes. Separate electrodes are used for control and treated groups. Measurement of pH is done with a meter equipped with a junction box and four glass combination electrodes. Separate electrodes are used for each treatment group.

Structural Variables. Populations of major taxa are determined according to the procedures outlined below.

Algae. Algal species are counted by tranferring a small sample from a stirred microcosm to a Palmer-Maloney counting slide. Species are typically grouped into 5 categories: Single Celled Algae, Diatoms, Non-Nitrogen Fixing Blue Green Algae, Nitrogen Fixing Blue Green Algae and Filamentous Green Algae. If test objectives dictate, finer or larger taxonomic groups may be used.

Zooplankton, Amphipods and Larval Insects. Amphipods are counted as the total number per microcosm. Zooplankton and insects are usually too abundant for total counts and must be subsampled by removing 50 mL. aliquots. The organisms in each aliquot are grouped into major categories (Cladocera, Copepoda, Ostracoda, Chironomidae) and counted. Aliquots are returned to the system.

Protozoa. Protozoa are counted by withdrawing 0.5 mL from each stirred microcosm and distributing it among 5 to 8 droplets on a glass plate. Each droplet is examined under 40X magnification. Protozoa are counted as flagellated, ciliated, and amoeboid forms.

Bacteria. Bacterial density can be determined using several techniques. To obtain a sample for each microcosm, inverted 10 mL pipettes, with a bulb on the tip are used. A combination sediment and water column sample is withdrawn from each microcosm by first collecting about 0.7 mL of sand-sediment from the bottom, then lifting the pipette while continuing to draw from the water column until the 10 mL mark on the pipette is reached. This results in a sample of about 13.2 mL of which 0.7 mL are sediment and 12.5 mL are liquid. The sample is transferred and temporarily stored in a sterile test tube. The choice of enumeration method will vary depending on the organism and the objectives of the test.

Data collection and analysis. Data collection and analysis have been

automated through a series of programs developed for use on an IBM PC or compatible computer. Functional measurements are entered directly into the computer and echoed to a printer as they are collected. Structural measurements are first recorded on data sheets and later entered into the computer. At the conclusion of an experiment, all data are combined into a single ASCII file which can be readily imported into a variety of commercial programs. For routine analyses we have developed a program to calculate means, standard deviations, and Dunnett's significant difference (15). The Dunnett's value is used to determine which of the treatments are different from the control (@p <= .05). This test is performed on each variable measured for each sampling day. It provides a determination of which variables are significantly affected and the duration of the effect.

From these calculations, the minimum observable and no-effect concentrations can be determined. From the periodic counts of the density of the microbial agent, determinations of the survival rate of the organism can also be reported. The calculations are visually summarized by plotting the means of each treatment group over time (Figure 1). An area corresponding to the upper and lower bounds of Dunnett's least significant difference ($p < .05$) is plotted around the line for the control mean. This envelope defines a region where the treatments are not significantly different from the control. Points lying outside this shaded area are significantly different ($p < .05$) from the control. We developed a program which automatically creates these plots on a Postscript^{tm} equipped laser printe:

III. QUALITY ASSURANCE

The sensitivity of the measured variables was determined by calculating a minimum detectable difference using the method given by Snedecor and Cochran (13). According to this method, the detectable difference (dd) is :

$$dd^2 = \frac{(10.5)\ s^2 2}{n}$$

where n is the sample size, s^2 is the variance and 10.5 is based on a paired, two-tailed design with a power of 90% and a significance level of $p \leq 0.05$.

Structural and functional measures differ widely in their sensitivity in a manner related to their background variability. Functional variables are by far the most sensitive with average minimum detectable differences in our tests ranging from 3 to 19%. These variables are used to select the final pool of microcosms after the development phase. Structural variables are considerably less sensitive. For the broadest taxonomic groupings (where the number of individuals was large and the sampling variability was low) detectable differences are approximately 50%. Detectable differences for rare taxa may be much higher, ranging well over 100%.

In tests with both chemicals and microorganisms, we have found

good agreement among experiments. Despite differences in community structure, the total densities of algae and zooplankton and the rates of primary production and community respiration attain similar levels in repeated experiments (L. J. Shannon, T. E. Flum, R. L. Anderson, and J.D. Yount; ASTM 12th Symposium on Ecotoxicolgy and Hazard Assessment, in press). Despite differences in species composition among repeated experiments, we have found that these microcosm systems develop in a predictable manner. The appropriate status of a microcosm ecosystem at the beginning of a test (after the six week development period) is summarized in Table 2. These values were derived from 150 microcosms used in our six most recent tests and should serve as a quality assurance guideline for determining whether microcosms have developed properly. Microcosms falling below these guidelines should not be used in tests.

IV. APPLICATIONS

As noted earlier, these microcosms have been used extensively for chemical testing. Recently they have been adapted for evaluating survival and effects of introduced microorganisms (L. J. Shannon, T.E. Flum, R.L. Anderson and J.D. Yount. ASTM 12th Symposium on Ecotoxicology and Hazard Assessment, in press). In an initial series of experiments, we tested spores and vegetative cells of (Bti). Recently we have evaluated Serratia marcescens, a non-spore former chosen because of its distinctive colony color. In all tests we were able to successfully track the microorganisms.

Survival of Introduced Microorganisms.

Bti is a registered pest control agent used in mosquito (Culicidae) and blackfly (Simuliidae) control programs. In our first microcosm test, commercially formulated Bti spores (Vectobac^{tm}; Abbott Laboratories) were added to microcosms at initial densities of 10^3, 10^4 and 10^6 CFU per mL. There were relatively few spore-formers among the "indigenous" microcosm microbial community, so we were able to monitor Bti spores by plating pasteurized samples of water and sediments on tryptic soy agar (TSA). Periodic observations indicated that Bti spores persisted at their inoculation densities for the duration of a six week monitoring period (Figure 2). Additional tests with another commercial formulation (Mosquito Attack^{tm}; Reuters Laboratories) and preparations of washed Bti spores from our laboratory confirmed this pattern of persistence.

If a test MPCA survives in a microcosm community it is important to determine how long it maintains its toxic or pathogenic properties. This can be accomplished either by periodically adding new target organisms to the microcosms and determining their survival, or by removing water and sediment samples from the microcosms and using them in bioassays against target organisms. Although Bti persisted in our tests, its ability to kill target crganisms quickly disappeared. Bioassays were conducted using water and sediment samples with first instar mosquito larvae, Aedes atropalpus, and consistently showed a complete loss of toxicity between 7 and 14 days post-application. This was not an unexpected result since several investigators have

noted a similar loss of Bti toxicity in natural systems (8, 11, 12, 19).

Serratia marcescens was used as a non-spore forming organism to provide a contrast to the Bti study. Serratia was readily monitored in microcosms, but in contrast to Bti it did not persist (Figure 3). This pattern of rapid decline is one of three patterns of bacterial survival noted in a recent review (1) and is probably more typical of bacterial introductions than the constant spore densities maintained in the Bti tests.

Movement of the test organism into the food chain. In monitoring the distribution of Bti, we looked at movement from the water and sediments to the biota. Our work at this point is preliminary, but results indicate that such measurements are possible. We examined Bti uptake in grazing zooplankton by removing 10 cladocerans and 10 copepods weekly. These animals were sonified in 5 ml of sterile water which was then pasteurized and plated on TSA agar. We found Bti accumulated rapidly in the animals, and was gradually lost over the four week test period (Figure 4).

Predicting the distribution of an introduced microorganism requires detailed studies of the distribution of the organism among the abiotic and biotic components of an ecosystem. Because of their small size, these microcosm systems contain somewhat limited zooplankton populations (typically 200 to 600 individuals). Repeated sampling of animals for microbial content

can severely deplete these populations, and result in changes in population densities which affect rates of primary production, respiration, nutrient cycling and other ecosystem processes. Distribution studies are probably best accomplished by using a large number of microcosms, and sacrificing complete systems at each sampling date. This will provide replication so that variability can be quantified, and avoid the possibility of confusing sampling-derived effects with true effects.

Ecological Effects. Bti did affect microcosm communities. At exposure levels of 10^3 spores per ml, all mosquito larvae and a few chironomids were killed within 24 hr. At the high exposure (10^6 spores per mL) chironomids were totally eliminated, but zooplankton and amphipods remained unaffected. Some functional measures also showed significant effects at the high exposure rate. The most consistent of these were brief increases in primary production (Figure 1) and community respiration. These changes apparently were a response to the loss of grazing species and the addition of nutrients associated with the Vectobac^{tm} formulation. Higher rates of community respiration probably reflected increased microbial activity in decomposing the carcasses of mosquitoes and chironomids and the organic materials used in the formulation. Primary production increased in response to both a loss of grazer species and an increased nutrient pool. The relatively short duration of these responses (approximately 2 weeks) indicated that long-term ecological consequences of Bti application would likely be minimal.

In testing an MPCA it is important to demonstrate that the

organism can function as a pest control agent under conditions found in the test system. If the test organism is not capable of operating under microcosm conditions, test results may have little relevance to natural systems where the agent is used. Our approach for MPCA testing is to incorporate a target animal into the test scheme. Aquatic target animals (e.g. mosquito larvae) can generally be added to the microcosm community. If these organisms are then killed by the test MPCA it demonstrates that the test organism is capable of functioning in the system, and that appropriate concentrations have been tested. The inclusion of target animals also allows for the detection of secondary effects arising from the loss of that species from the test community.

V. INTERPRETATION

Statistical and ecological significance are not necessarily equivalent. As noted earlier, it is important to know the statistical properties of the variables being monitored. In these microcosms functional measurements are quite sensitive and may show statistically significant differences for deviations from the control of as little as 5 to 10%. Such changes may be statistically significant, but their ecological significance is probably small. With less sensitive variables (e.g. measures of population density) where deviations of 50% or greater are necessary to show statistical significance, significant statistical effects are likely to be ecologically important. At the extreme end of the scale some measurements have such a high degree of inherent variability, (e.g. protozoan densities)

that it is difficult to demonstrate statistical significance, even though there may have been large changes in populations and ecologically significant differences could have occurred. It is evident that both an understanding of the statistical properties of a system and a knowledge of ecological relationships will be necessary to predict whether an introduced microorganism will cause a significant ecological effect.

There is more to consider in making a judgment of whether an observed effect is ecologically significant. Beyond a determination of which variables were affected and the statistical properties of those variables, the ability and time required for a microcosm community to recover from perturbation should also be considered. A great advantage of microcosm tests is their ability to simulate immigration and recolonization thereby affording the opportunity to demonstrate recovery. Disturbances, even major ones, from which microcosms can recover (i.e. return to control levels) perhaps have less ecological significance than those from which they fail to recover. It is important that the ability of the microcosm community to recover (its resilience) be used in evaluating the ecological significance of observed effects. In our experience with Bti, most affected variables recovered to control levels by the end of the test, providing evidence for a prediction that the ecological consequences of Bti application are relatively minor.

We have to compare data from microcosm tests to data from natural systems tests. In our first evaluation we compared responses from microcosm tests with the insecticide chlorpyrifos

(Dursban^{tm}) to those observed in concurrent outdoor tests conducted in the littoral zone of a pond (Siefert, R.E., Project Leader, USEPA,ERL-Duluth, Duluth, MN, 1988). The results showed that untreated (control) microcosms had many functional and structural similarities to the field sites, indicating that the microcosms were accurate models of shallow ponds. Microcosm responses to chlorpyrifos were similar to those observed in the field. In both laboratory and in the field, amphipods and cladocerans were lost at 0.5 ug/L of chlorpyrifos, while copepods and ostracods suffered less severe reductions. Algae were not directly affected in either system.

The need for a system that will allow prediction of effects and survival of novel microorganisms was outlined earlier. More comparisons of microcosm and field data are necessary, but on the basis of this comparison and others (16, F.S. Stay, A. Katko, C.M. Rohm, M.A. Fix, and D.P. Larson, Arch. Environ. Contam. Toxicol., in press), it appears that microcosms may play a significant role in the early detection of field effects of chemicals. It is logical that effect and survival evaluations could utilize microcosms in developing an assessment of the risk of introduced microorganisms.

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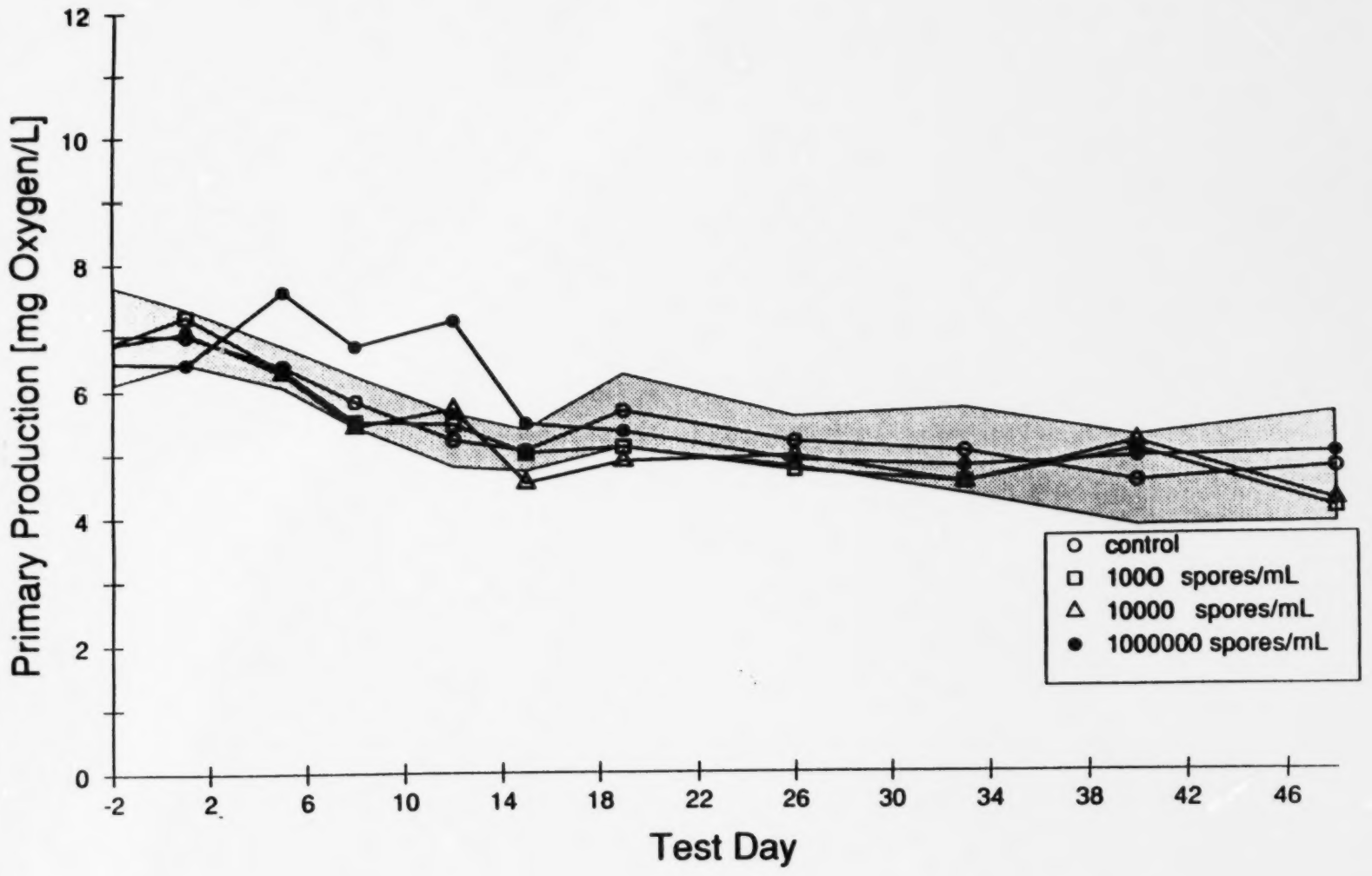
Figure Legends

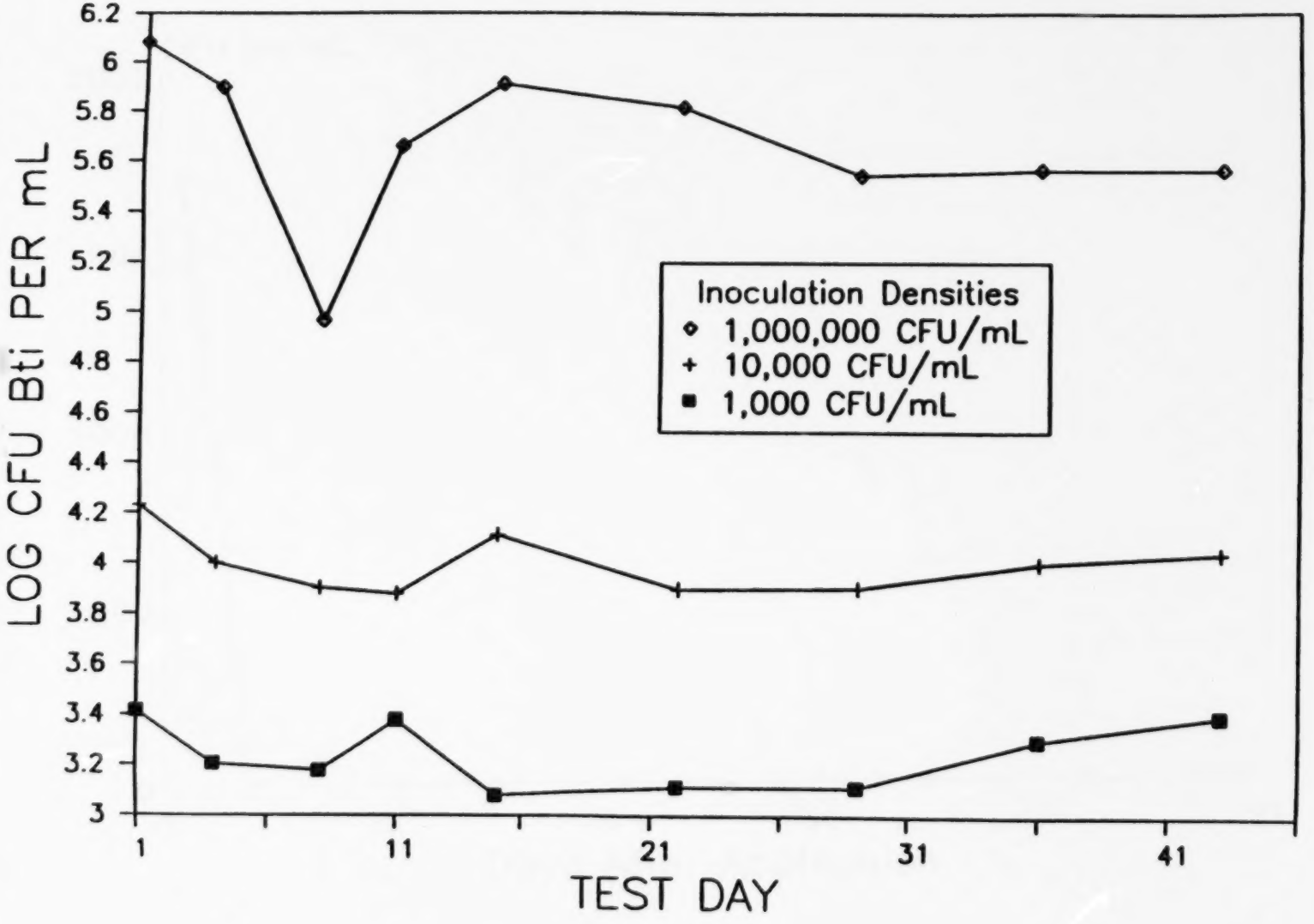
1. The effect of Bti (Vectobac^{tm}, Abbott Laboratories) application on primary production (as mg/L O_2 per 12 hr) in microcosm communities. Each point represents the mean of 5 replicate microcosms. The shaded envelope represents Dunnett's significant difference from the control ($p \leq 0.05$). Bti added on day 0.

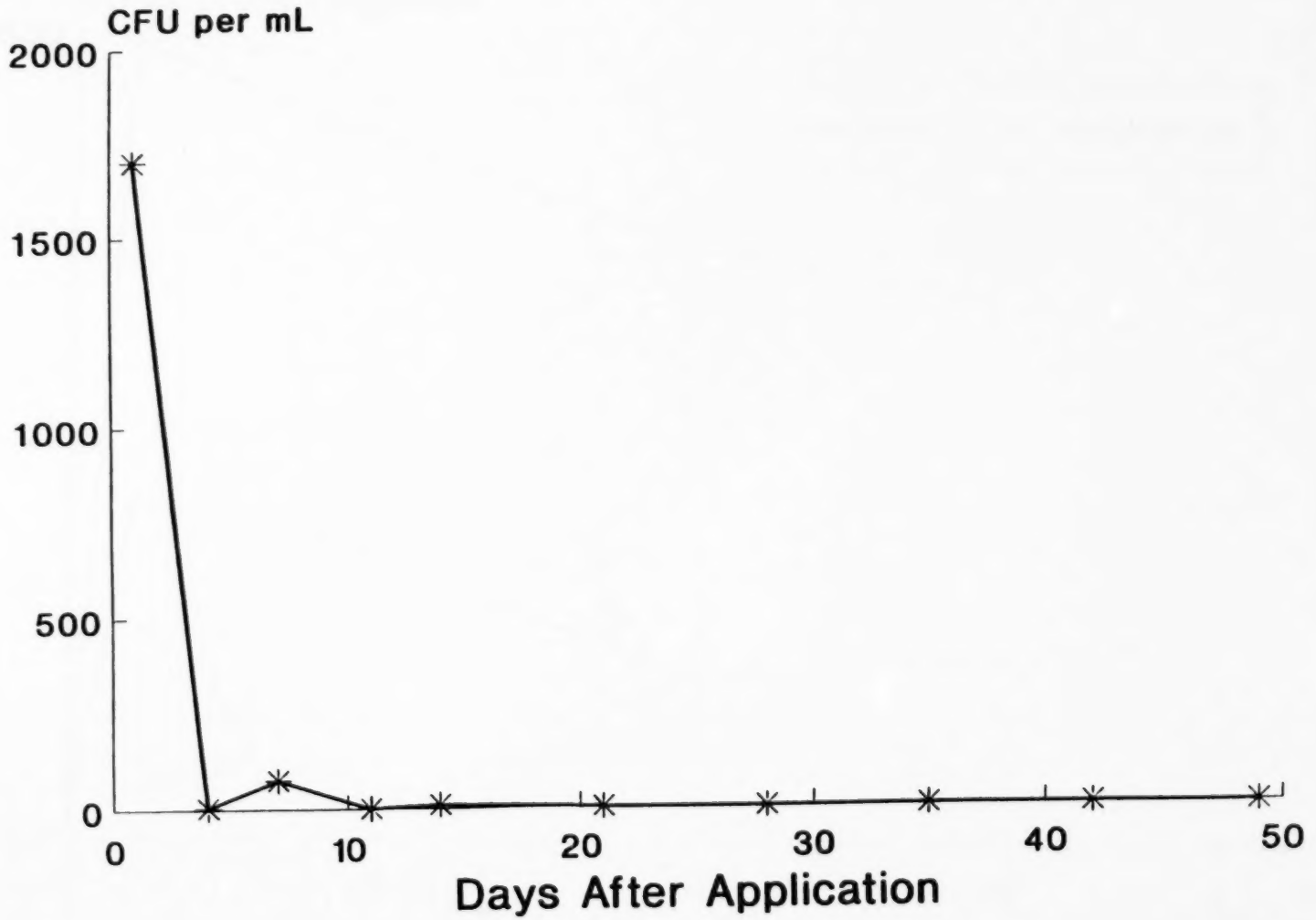
2. Survival of Bti (Vectobac^{tm}, Abbott Laboratories) in microcosms. Counts are based on pasteurized samples plated on TSA agar.

3. Survival of Serratia marcesens in microcosms.

4. Uptake of Bti (Mosquito Attack, Reuters Laboratories) by crustacean zooplankton in microcosm communities. Each point represents the mean of 10 animals.


Primary Production [mg Oxygen/L]
12
10
8
6
4
2
0
-2
2
6
10
14
18
22
26
30
34
38
42
46
Test Day
control
1000 spores/mL
10000 spores/mL
1000000 spores/mL


LOG CFU Bti PER mL
6.2
6
5.8
5.6
5.4
5.2
5
4.8
4.6
4.4
4.2
4
3.8
3.6
3.4
3.2
3
1
11
21
31
41
TEST DAY
Inoculation Densities
1,000,000 CFU/mL
10,000 CFU/mL
1,000 CFU/mL


CFU per mL
2000
1500
1000
500
0
0
10
20
30
40
50
Days After Application

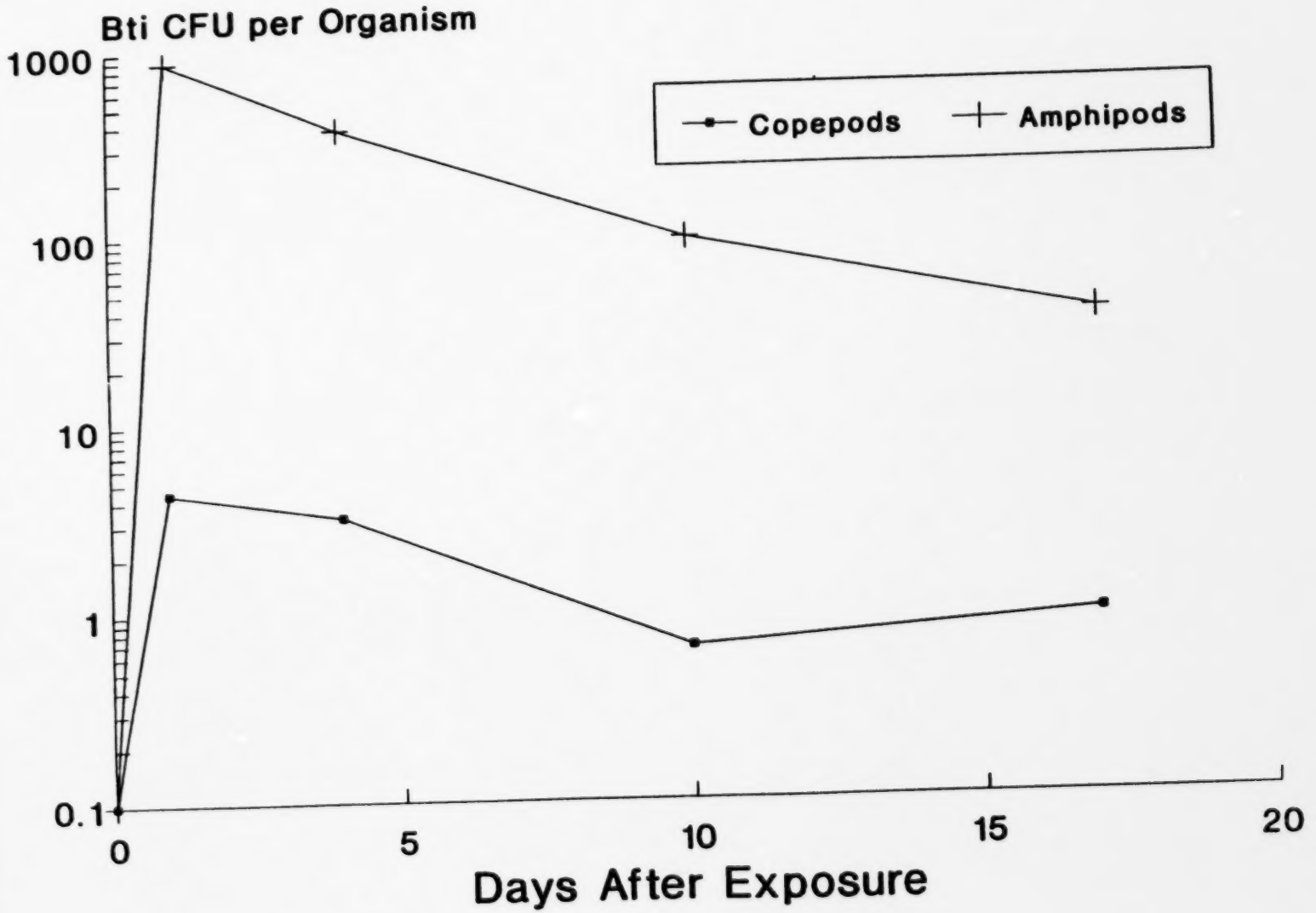

Bti CFU per Organism
1000
100
10
1
0.1
Copepods
Amphipods
0
5
10
15
20
Days After Exposure

Table 1: History of tests conducted in Mixed Flask Microcosms.

Compound	No. of Tests
I. Original Leffler Protocol	
Copper Sulfate - $CuSO_4$	(2)
1-Octanol	(2)
1-Decanol	(2)
1-Hexanol	(2)
Aniline	(1)
2,6 - Diisopropylaniline	(1)
4-Hexyloxyaniline	(1)
2,3,4,6-Tetrachloroaniline	(2)
2-(Octyloxy) Acetanilide	(1)
Diuron	(2)
Salicyclanilide	(2)
Hercofloc 863	(1)
Hercofloc 872	(1)
Atrazine	(4)
Fluorene	(4)
II. Shannon (MFC) Protocol	
Dursban	(2)
Fenvalerate	(1)
Bacillus thuringiensis subsp.*israelensis*	(3)
Serratia marcesens	(1)

Table 2:
Mean values for major microcosm variables at the sixth week of the development phase.

	AM pH	Oxygen Gain	Oxygen Loss	P/R	Total Zooplankton per liter	Total Algae per mL.	Total Protozoa per mL.
Mean	8.49	4.40	4.61	0.97	255	2.1×10^8	35
SD	0.59	1.01	1.05	0.13	138	1.5×10^8	25